# $CB_1$ Receptor Antagonist SR141716A Inhibits $Ca^{2+}$-Induced Relaxation in $CB_1$ Receptor–Deficient Mice

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***Abstract***—Mesenteric branch arteries isolated from cannabinoid type 1 receptor knockout ($CB_1^{-/-}$) mice, their wild-type littermates ($CB_1^{+/+}$ mice), and C57BL/J wild-type mice were studied to test the hypothesis that murine arteries undergo high sensitivity $Ca^{2+}$-induced relaxation that is $CB_1$ receptor dependent. Confocal microscope analysis of mesenteric branch arteries from wild-type mice showed the presence of $Ca^{2+}$ receptor–positive periadventitial nerves. Arterial segments of C57 control mice mounted on wire myographs contracted in response to 5 $\mu$mol/L norepinephrine and responded to the cumulative addition of extracellular $Ca^{2+}$ with a concentration-dependent relaxation that reached a maximum of 72.0±6.3% of the prerelaxation tone and had an $EC_{50}$ for $Ca^{2+}$ of 2.90±0.54 mmol/L. The relaxation was antagonized by precontraction in buffer containing 100 mmol/L $K^+$ and by pretreatment with 10 mmol/L tetraethylammonium. Arteries from $CB_1^{-/-}$ and $CB_1^{+/+}$ mice also relaxed in response to extracellular $Ca^{2+}$ with no differences being detected between the knockout and their littermate controls. SR141716A, a selective $CB_1$ antagonist, caused concentration-dependent inhibition of $Ca^{2+}$-induced relaxation in both the knockout and wild-type strains (60% inhibition at 1 $\mu$mol/L). O-1918, a cannabidiol analog, had a similar blocking effect in arteries of both wild-type and $CB_1^{-/-}$ mice at 10 $\mu$mol/L. In contrast, 1 $\mu$mol/L SR144538, a cannabinoid type 2 receptor antagonist, or 50 $\mu$mol/L 18$\alpha$-glycyrrhetinic acid, a gap junction blocker, were without effect. SR141716A (1 to 30 $\mu$mol/L) was also assessed for nonspecific actions on whole-cell $K^+$ currents in isolated vascular smooth muscle cells. SR141716A inhibited macroscopic $K^+$ currents at concentrations higher than those required to inhibit $Ca^{2+}$-induced relaxation, and appeared to have little effect on currents through large conductance $Ca^{2+}$-activated $K^+$ channels. These data indicate that arteries of the mouse relax in response to cumulative addition of extracellular $Ca^{2+}$ in a hyperpolarization-dependent manner and rule out a role for $CB_1$ or $CB_2$ receptors in this effect. The possible role of a nonclassical cannabinoid receptor is discussed. **(*Hypertension*. 2002;39:251-257.)**

**Key Words:** mesenteric arteries ■ calcium ■ relaxation ■ potassium channels

Over the past several years, a novel, extracellular $Ca^{2+}$-activated, vasodilator pathway has been described in isolated rat arteries. The current model describing this system holds that elevation of extracellular $Ca^{2+}$, as occurs in the interstitial compartments of some tissues, activates a $Ca^{2+}$-sensing receptor localized on perivascular sensory nerves. Activation of this receptor causes the release of a transmitter that diffuses to underlying vascular smooth muscle and induces hyperpolarization-mediated relaxation through activation of a vascular smooth muscle cannabinoid type 1 ($CB_1$) receptor.[1] Among the findings that support this pathway are the demonstration that dorsal root ganglia and perivascular sensory nerves express a protein that is immunoreactive with antibodies raised against the parathyroid $Ca^{2+}$ receptor[2] and that the magnitude of $Ca^{2+}$-induced relaxation positively correlates with $Ca^{2+}$ receptor–positive nerve density.[3] The relaxation is inhibited by nonselective antagonism of potassium ($K^+$) channel activity with precontraction in depolarizing concentrations of KCl and pretreatment with tetraethyammonium[2] and by selective blockade of large conductance $K_{Ca}$ channels with iberiotoxin[4] and charybdotoxin.[5] Additional work has shown that the relaxation is blocked by the selective $CB_1$ receptor antagonist SR141716A and is mimicked by anandamide,[4] an endocannabinoid, which has been shown to be formed in neuronal tissue.[6,7]

Over the past 2 years, several reports have appeared that indicate that in addition to its high potency as a $CB_1$ receptor antagonist,[8] at concentrations of ≥10 $\mu$mol/L, SR141716A also has activity as an antagonist of type 2 cannabinoid ($CB_2$) receptors,[8] $Ca^{2+}$ entry channels,[9] $K_{Ca}$ channels,[9] and gap junctions.[10] Although we have found that lower concentrations of SR141716A inhibit the mesenteric vasodilator re-

Received June 15, 2001; first decision July 17, 2001; revision accepted October 11, 2001.

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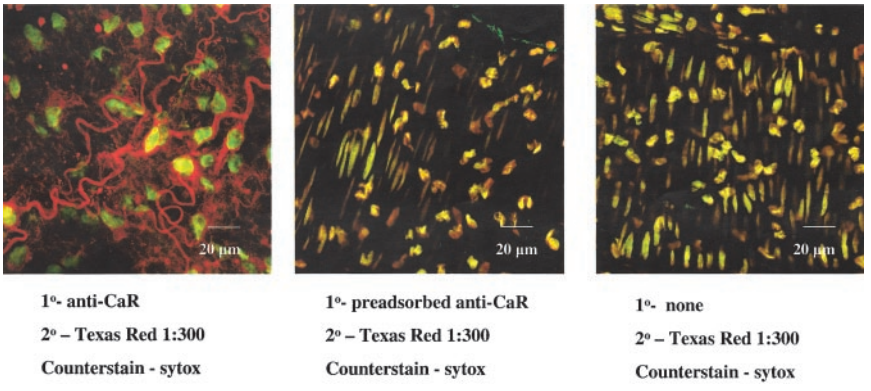


**Figure 1.** 3D confocal images of mesenteric branch arteries of wild-type C57BL/6 mice. Left, $Ca^{2+}$ receptor–positive nerves in the adventitial layer and nuclei of adjacent cells. Middle, Reduction in nerve staining after preadsorption of primary antibody with excess antigen. Right, Absence of staining when primary antibody is omitted from the assay. Note that middle and right panels contain oblong nuclei from smooth muscle cells in the tunica media. Photos were taken with a 63× water immersion objective.

sponse to anandamide,[4] we believed it was important to obtain additional information regarding whether $Ca^{2+}$-induced relaxation is mediated by the $CB_1$ receptor. The $CB_1^{-/-}$ mouse[11] offers a direct means of testing the hypothesis that $Ca^{2+}$-induced relaxation is mediated by a transmitter with activity at the $CB_1$ receptor. We have therefore performed experiments to (1) determine whether arteries isolated from mice undergo extracellular $Ca^{2+}$-induced relaxation and whether the relaxation shares properties with the relaxation observed in rats and (2) to test the hypothesis that $Ca^{2+}$-induced relaxation is mediated by the $CB_1$ receptor.

## Methods

### Animals

All experiments using animals were approved by the appropriate institutional animal care and use committees. $CB_1$ receptor knockout ($CB_1^{-/-}$) mice and homozygous littermate controls ($CB_1^{+/+}$) were bred from founders that were generously provided by Dr Andreas Zimmer (Rheinische Friedrich-Wilhelms-Universitat Klinik und Poliklinik fur Psychiatrie und Psychotherapie Abteilung Molekulare Neurobiologie, Bonn, Germany) and that have been described in detail.[11] Some control experiments were performed using tissue obtained from nonlittermate inbred C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, Ind). Male and female animals were studied, and mesenteric tissue was isolated after the animals were killed by cervical dislocation. Male golden Syrian hamsters (9 to 12 weeks of age) were obtained from Charles Rivers Laboratories (Wilmington, Mass) and on the day of the experiment were killed by $CO_2$ asphyxiation followed by cervical dislocation. Cremaster muscles were removed, and second- and third-order arterioles were isolated by hand dissection as described previously.[12]

### Immunocytochemistry

Mesenteric branch arteries were microdissected from isolated mesenteries and prepared for immunostaining as described previously.[2] Fixed and blocked segments were incubated overnight with a polyclonal anti-$Ca^{2+}$ receptor antibody (1:50, Affinity Bioreagents) in the presence or absence of excess antigen. Anti-rabbit IgG conjugated with Texas Red (1:300, Molecular Probes) was used as the secondary antibody. Vessel segments were viewed using a Zeiss LSM 510 confocal microscope and 63× water immersion objective (Zeiss Instruments).

### Biophysical Measurements

Isometric force generation was measured using previously described methods.[2] Relaxation to $Ca^{2+}$ or acetylcholine was assessed by cumulatively adding the agent to vessels that had been precontracted to a steady-state tension using 5 μmol/L norepinephrine (NE). The percentage of relaxation was calculated taking the magnitude of the prerelaxation tone as 100% of the amount that the vessel could relax. When the effect of an antagonist, ie, SR141716A, was assessed on the relaxation response, the vessel was pretreated with the compound for 10 to 15 minutes. Afterward, the contraction was induced by the addition of NE, and the response to the dilator assessed.

An expanded Biophysical Measurements section can be found in an online data supplement available at http://www.hypertensionaha.org.

### Electrophysiology

Vascular smooth muscle cells were enzymatically isolated from hamster cremasteric arterioles using a papain digestion medium as described previously.[12] Macroscopic $K^+$ currents were measured using the conventional whole cell recording method.[13] Currents were measured, and membrane potential was clamped with a Warner PC-505A patch-clamp amplifier (Warner Instrument Corp), filtered at 1 kHz, and sampled at 5 kHz. As reported previously, all currents were normalized to cell capacitance to account for any differences in cell size. Cells were held at −60 mV and then stepped, for 400 ms, to test potentials from −90 to 60 mV in 10-mV increments. The average current during the last 200 ms of the test pulse was then measured at each potential and used to construct current/voltage relationships.

An expanded Electrophysiology section can be found in an online data supplement available at http://www.hypertensionaha.org.

### Materials

SR141716A and SR144538 were obtained from Sanofi Recherche and dissolved in absolute ethanol; O-1918 was synthesized by Dr Raj Razdan (Organix Inc, Woburn, Mass) and dissolved in ethanol. All other chemicals were of reagent grade or better and obtained from Sigma Chemical or other commercial suppliers, as dictated by the purchasing regulations of the University of North Carolina System.

### Statistical Analysis

The agonist concentration eliciting 50% of the maximal relaxation ($EC_{50}$) was determined from plots of the percentage of initial tension versus the concentration of agonist. All data are presented as mean±SEM, and statistical analysis was performed using the SYSTAT software package. Comparisons among groups were performed using ANOVA with a repeated measures design when appropriate. A value of $P<0.05$ was taken to indicate a statistically significant difference.

## Results

Whole-mount sections of mouse mesenteric branch arteries were stained with anti-$Ca^{2+}$ receptor antibody and viewed using a laser confocal microscope (Figure 1). Analysis of sections from at least 6 mice showed that nerve fibers located in the adventitia stained positively for $Ca^{2+}$ receptor protein, and $Ca^{2+}$ receptor–positive staining was not present elsewhere in the vessel wall (Figure 1A and 1B). Moreover, preincubation of the primary antibody with blocking peptide

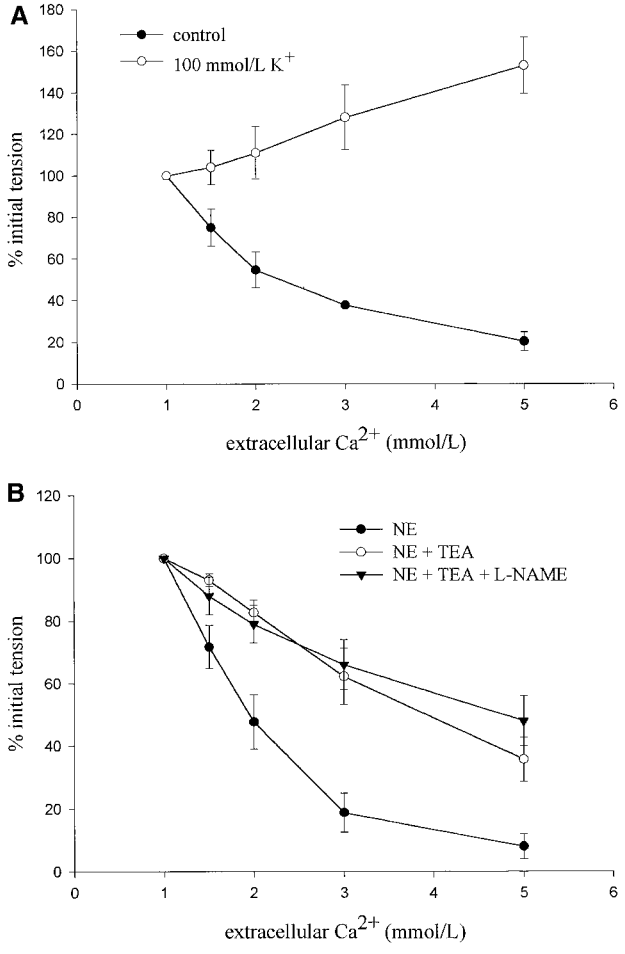


**Figure 2.** Response of mesenteric branch arteries of wild-type C57BL/6 mice to the cumulative addition of extracellular $Ca^{2+}$ after precontraction with 5 $\mu$mol/L NE or 100 mmol/L $K^+$ (A) or after precontraction with 5 $\mu$mol/L NE and pretreatment with 10 mmol/L TEA or a mixture of 10 mmol/L TEA and 0.3 mmol/L $N^G$-nitro-L-arginine methyl ester (L-NAME, B). Values are mean±SEM, $*P<0.05$ vs NE control, n=3 to 5 per group.

or staining with secondary antibody alone eliminated nerve staining (Figure 1C).

Mesenteric branch arteries isolated from C57 control mice and precontracted with 5 $\mu$mol/L NE relaxed in response to cumulative addition of extracellular $Ca^{2+}$, with a maximal relaxation response of 72.0±6.3% of the prerelaxation tone (n=6) and an $EC_{50}$ for $Ca^{2+}$ of 2.9±0.54 mmol/L (Figure 2A). To learn whether $Ca^{2+}$-induced relaxation of mouse arteries is dependent on functional $K^+$ channels, the relaxation response was also determined in vessels precontracted with 100 mmol/L $K^+$ or pretreated with 10 mmol/L tetraethylammonium (TEA). Precontraction with a depolarizing concentration of $K^+$ converted the $Ca^{2+}$-induced relaxation into a $Ca^{2+}$-induced contraction (Figure 2A). Moreover, pretreatment with 10 mmol/L TEA significantly inhibited the relaxation induced by cumulative addition of extracellular $Ca^{2+}$ (Figure 2B). Because a residual relaxation to $Ca^{2+}$ was observed in the presence of 10 mmol/L TEA, we tested the hypothesis that this component is mediated by NO. Pretreatment with 0.3 mmol/L $N^G$-nitro-L-arginine methyl ester, however, was without further inhibitory effect (Figure 2B).

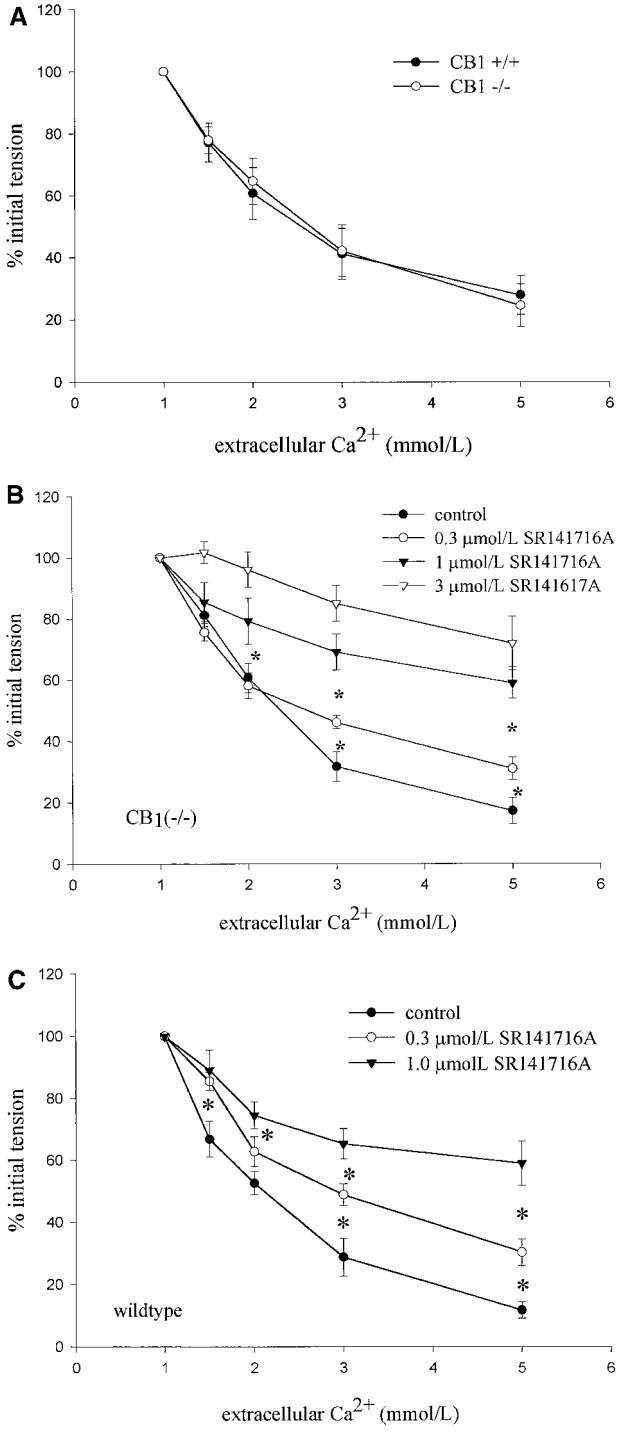


**Figure 3.** A, Response of mesenteric branch arteries isolated from $CB_1^{+/+}$ and $CB_1^{-/-}$ mice to the cumulative addition of extracellular $Ca^{2+}$ after precontraction with 5 $\mu$mol/L NE. Values are mean± SEM, n=6; no significant differences were detected. Effect of 0.3 and 1.0 $\mu$mol/L SR141716A on $Ca^{2+}$-induced relaxation of mesenteric branch arteries isolated from $CB_1^{-/-}$ mice (B) and wild-type control mice (C). Values are mean± SEM, n=4 to 6. $*P<0.05$ between points.

Branch arteries isolated from $CB_1^{-/-}$ animals and $CB_1^{+/+}$ littermates were also studied. $Ca^{2+}$ caused a concentration-dependent relaxation of $CB_1^{-/-}$ arteries that was not different from that observed in vessels isolated from $CB_1^{+/+}$control

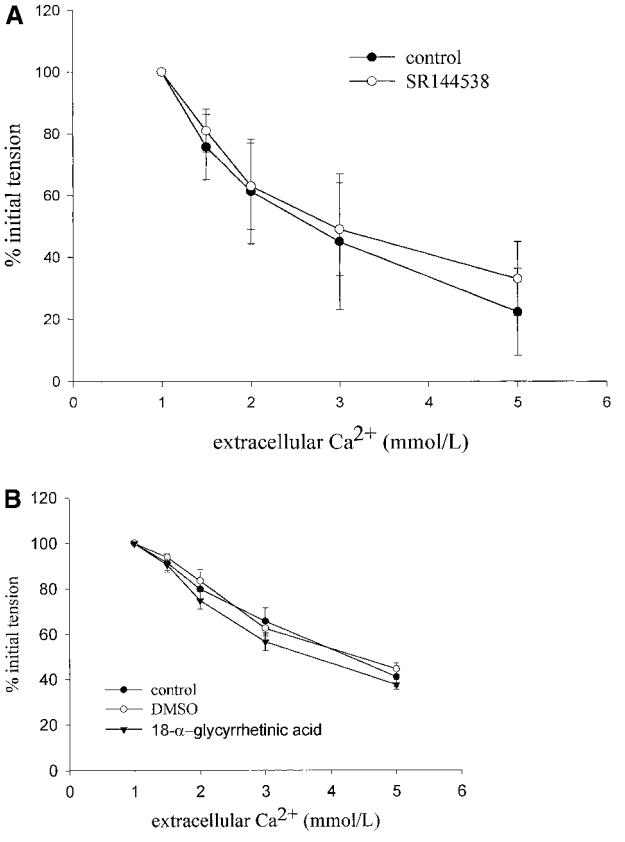


**Figure 4.** Effect of pretreatment with 3 μmol/L SR144538 on $Ca^{2+}$-induced relaxation of arteries isolated from $CB_1^{-/-}$ mice (A) or effect of preincubation of the artery with 50 μmol/L 18α-glycyrrhetinic acid on arteries isolated from wild-type C57BL/6 mice (B). Values are mean±SEM, n=3 to 4. No effect of either antagonist was detected.

animals (maximal relaxation response, 75.5±6.9%, $P=0.954$; $EC_{50}$, 3.2±0.28 mmol/L, n=6, $P=0.714$) (Figure 3A). The response of $CB_1^{+/+}$ and $CB_1^{-/-}$arteries to the cumulative addition of acetylcholine was also assessed. Acetylcholine induced concentration-dependent relaxation of arteries isolated from both the wild-type and knockout strains (data not shown). No differences in either the maximal response or the sensitivity of the arteries to acetylcholine were detected (maximal $CB_1^{+/+}$ response versus $CB_1^{-/-}$, 76.9±7.5% versus 78.3±10.7%, n=3 and n=4, respectively, $P=0.998$; $EC_{50}$ $CB_1^{+/+}$ versus $CB_1^{-/-}$, 0.71±0.42 versus 0.21±0.15 μmol/L, $P=0.888$).

Preincubation of vessels with increasing concentrations of the $CB_1$ antagonist SR141716A dose-dependently inhibited $Ca^{2+}$-induced relaxation of NE-precontracted arteries isolated from both the $CB_1^{-/-}$ strain (Figure 3B) and the $CB_1^{+/+}$ control (Figure 3C). These data indicate that the threshold for SR141617A inhibition of $Ca^{2+}$-induced relaxation is <300 nmol/L and that the $IC_{50}$ lies between 300 nmol/L and 1 μmol/L.

To learn whether the blockade by SR141716A might be mediated by antagonist activity at the $CB_2$ receptor, we assessed the effect of pretreatment with the selective $CB_2$ antagonist SR144538.[14] Pretreatment with 1 μmol/L SR144538 had no effect on relaxation induced by $Ca^{2+}$ in arteries isolated from either the knockout or wild-type animals (Figure 4A). To assess the possible role of gap junctional activity in the relaxation response, the effect of preincubation with 18α-glycyrrhetinic acid (50 μmol/L), a gap junction blocker[10] was assessed. 18α-glycyrrhetinic acid had no effect on the relaxation response determined in vessels from 4 animals (Figure 4B). Our previous data indicate that $Ca^{2+}$-induced relaxation is inhibited by iberiotoxin, a selective inhibitor of large conduct $K_{Ca}$ channels.[4] To learn whether a possible nonselective effect of SR141716A at $K^+$ channels plays a role in the inhibition of $Ca^{2+}$-induced relaxation, we assessed the effect of a range of concentrations of SR141716A on whole-cell $K^+$ channel currents in isolated vascular smooth muscle cells. SR141716A inhibited macroscopic $K^+$ currents in a concentration-dependent fashion, (Figure 5A), with maximal effects observed at 10 μmol/L (Figure 5B). To learn whether SR141716A blocks $K_{Ca}$ channel activity, we tested whether the inhibitory action of the

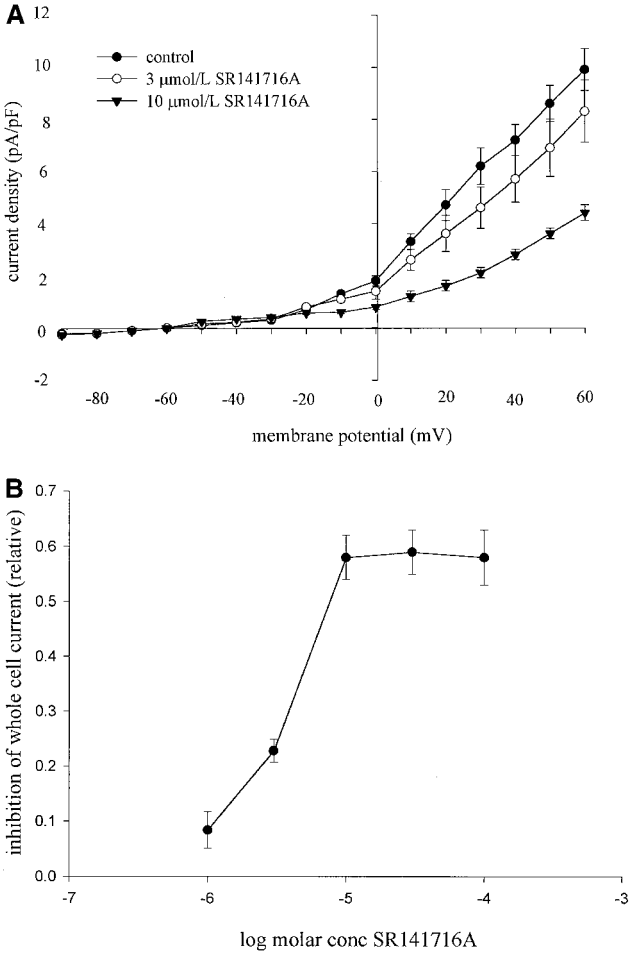


**Figure 5.** A, Effect of SR141716A on whole-cell $K^+$ currents in isolated vascular smooth muscle cells under control conditions or in the presence of 3 and 10 μmol/L SR141716A. Values are mean current densities±SEM, n=5 to 6. Both concentrations significantly inhibited currents ($P<0.05$). B, Concentration response data for the inhibitory effect of SR141716A on whole-cell $K^+$ currents. Values are mean inhibition±SEM, n=3 to 13, measured by stepping to 60 mV from a holding potential of −60 mV. SR141716A inhibited the currents in a concentration-dependent fashion between 1 and 10 μmol/L.

antagonist was additive with respect to TEA. Initial experiments showed that TEA (1 mmol/L) and iberiotoxin (100 nmol/L) inhibit whole-cell $K^+$ channel currents to a similar degree and in a nonadditive fashion (Figure 6A). In contrast, the $K^+$-blocking effect of SR141716A was clearly additive to the inhibition produced by 1 mmol/L TEA (Figure 6B and 6C) or 100 nmol/L iberiotoxin (n=3, data not shown). These observations suggest that SR141716A has little effect on currents through large conductance $K_{Ca}$ channels.

A previous report[15] from one of our laboratories (G.K.) showed that abnormal-cannabidiol induces relaxation of the isolated perfused mesenteric artery bed of the rat and mouse and that the relaxation is blocked by cannabidiol and by SR141716A. We therefore assessed the effect of a novel cannabidiol analog, O-1918, that has been found to block abnormal cannabidiol-induced relaxation in isolated rat arteries (S. Batkai and G. Kunos, 2001, unpublished observations). Pretreatment of arteries isolated from C57BL/6 wild-type mice and $CB_1^{-/-}$ mice with 10 $\mu$mol/L O-1918 for 15 minutes caused significant inhibition of $Ca^{2+}$-induced relaxation (Figure 7A and 7B), whereas it was without effect on relaxation induced by acetylcholine (Figure 7C).

## Discussion

The present studies were performed to determine whether arteries isolated from mice undergo extracellular $Ca^{2+}$-induced relaxation, to learn whether the relaxation shares properties with $Ca^{2+}$-induced relaxation observed in rats, and to test the hypothesis that $Ca^{2+}$-induced relaxation is mediated by the $CB_1$ receptor. The major new findings are that (1) murine mesenteric branch arteries are supplied with $Ca^{2+}$ receptor–positive periadventitial nerves and relax in response to the cumulative addition of extracellular $Ca^{2+}$; (2) the relaxation is blocked by conditions that inhibit $K^+$ channel–dependent relaxation; (3) the relaxation occurs in the absence of $CB_1$ receptors; and (4) the relaxation is antagonized by SR141716A in arteries of the $CB_1$ receptor knockout mouse.

Earlier studies, performed using arteries isolated from the rat, showed that when care is taken to preserve the integrity of the vascular adventitia, cumulative addition of extracellular $Ca^{2+}$ causes concentration-dependent relaxation.[2] In recent studies of arterial segments mounted on 14-$\mu$m gold wires, we have found $EC_{50}$ values for $Ca^{2+}$ of 1.45±0.02 mmol/L (R.D. Bukoski, 2001, unpublished observations), which is well within the range of the concentrations that have been shown to occur in the interstitial fluid compartment of the small bowel[16] and renal cortex.[17] The relaxation is attenuated by sensory denervation and correlates with the presence of $Ca^{2+}$ receptor–positive nerve fibers.[2] $Ca^{2+}$-induced relaxation of rat arteries has also been shown to be inhibited by blockers of the large conductance $K_{Ca}$ channel[4,5] and by SR141716A[4], a compound with $CB_1$ antagonist activity.[8] Moreover, the relaxation is mimicked by anandamide, an endocannabinoid $CB_1$ receptor agonist[18] with hyperpolarizing vasodilator activity.[19–21] Together, these observations have led to the hypothesis that changes in the concentration of $Ca^{2+}$ in the interstitial fluid compartment are sensed by a sensory nerve $Ca^{2+}$ receptor, and activation of this receptor is coupled with

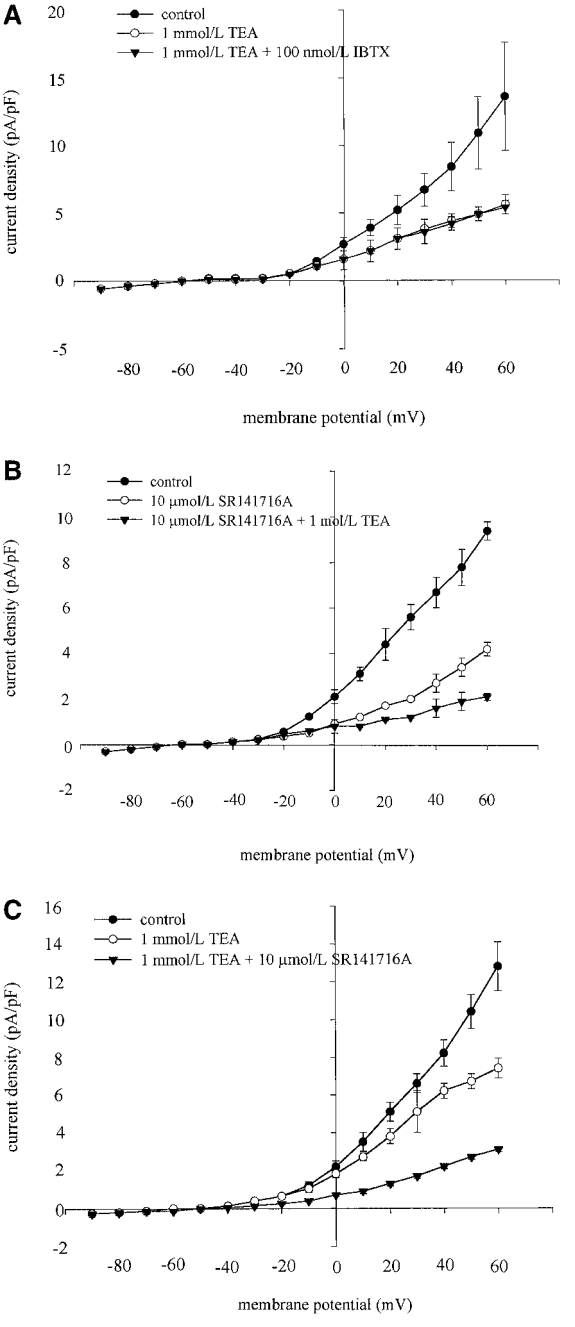


**Figure 6.** Inhibition of whole-cell $K^+$ currents by SR141716A is additive with TEA. A, Values are mean current density±SEM (n=5) in the absence (control) or presence of either 1 mmol/L TEA or 1 mmol/L TEA and 100 nmol/L iberiotoxin. Note that iberiotoxin produced no additional inhibition to that produced by TEA, indicating that TEA was maximally blocking currents through large conductance $K_{Ca}$ channels. B, Values are mean current densities±SEM (n=6) in the absence (control) or presence of either 10 $\mu$mol/L SR141716A or 10 $\mu$mol/L SR141716A and 1 mmol/L TEA. TEA caused additional inhibition of currents above that produced by SR141716A. C, Values are mean current densities±SEM (n=5) in the absence (control) or presence of either 1 mmol/L TEA or 1 mmol/L TEA and 10 $\mu$mol/L SR141716A. SR141716A produced additional inhibition in addition to that produced by TEA, indicating that SR141716A is inhibiting currents through channels other than large-conductance $K_{Ca}$ channels.

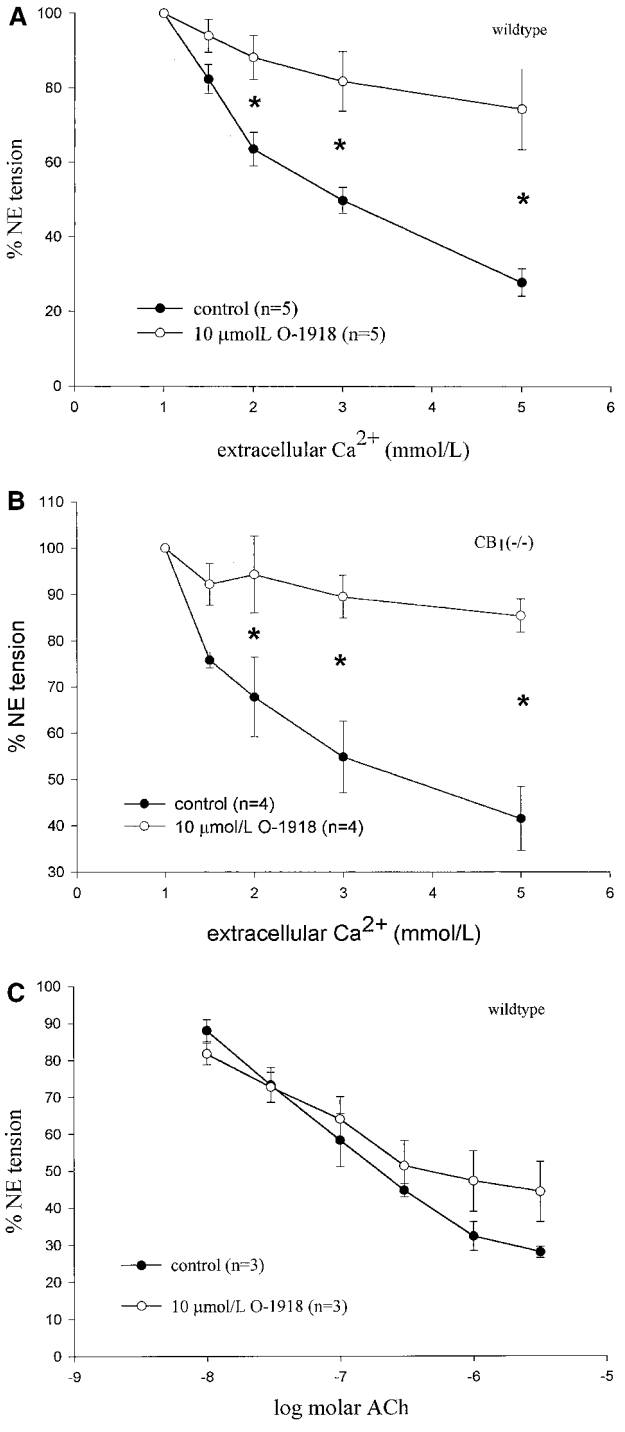


**Figure 7.** Effect of pretreatment with 10 $\mu$mol/L of the cannabidiol analog O-1918 on $Ca^{2+}$-induced relaxation of arteries isolated from C57BL/6 mice (A) and $CB_1^{-/-}$ mice (B). Values are mean±SEM, n=4 or 5 per group, $^*P < 0.05$. C, Acetylcholine-induced relaxation in arteries of C57BL/6 mice. Values are mean±SEM, n=3 per group. No differences were detected.

the release of an endocannabinoid-like hyperpolarizing vasodilator transmitter with activity at the $CB_1$ receptor.[1]

The present studies demonstrate that perivascular nerves of mouse mesenteric branch arteries express $Ca^{2+}$ receptor protein. These findings extend our prior demonstration that dorsal root ganglia of the rat express cDNA encoding a protein that is homologous with rat kidney $Ca^{2+}$ receptor[2]; that DRG and mesenteric arteries express a protein that is immunoreactive with the parathyroid $Ca^{2+}$ receptor[2]; and that arteries isolated from the mesentery, kidney, and brain of the rat express $Ca^{2+}$ receptor–positive protein.[22] As in the rat, $Ca^{2+}$-induced relaxation of the mouse mesenteric artery is completely blocked by precontraction of the vessel segment with a depolarizing concentration of potassium chloride and is significantly attenuated by blockade of $K^+$ channel activity with 10 mmol/L TEA.[2] These observations suggest the involvement of $K^+$ channels and a hyperpolarization-dependent mechanism of relaxation. To our knowledge, this is the first demonstration of $Ca^{2+}$ receptor expression in perivascular nerves and high sensitivity $Ca^{2+}$-induced relaxation of a species other than the rat.

The second new finding of the present study is the observation that $Ca^{2+}$-induced relaxation occurs in mesenteric branch artery segments isolated from $CB_1$ receptor knockout mice. These experiments were designed to follow up on the prior observation that $Ca^{2+}$-induced relaxation in the rat is blocked by SR141716A and to directly test the hypothesis that $Ca^{2+}$-induced relaxation requires an active $CB_1$ receptor. The results clearly indicate that the $CB_1$ receptor is not involved in the $Ca^{2+}$-induced relaxation event in the mouse.

A third finding that merits discussion is our observation that $Ca^{2+}$-induced relaxation of mouse artery is inhibited by SR141716A. This supports our earlier finding obtained using the rat.[4] In addition, the observation that the sensitivity of $Ca^{2+}$-induced relaxation to inhibition by SR141716A is similar in arteries isolated from the $CB_1^{-/-}$ knockout and wild-type control argues against the possibility that SR141716A inhibits $Ca^{2+}$-induced relaxation via $CB_1$ receptors in wild-type mice, but suggests that a different site is involved in the inhibition observed in $CB_1$ receptor knockout mice.

As noted in the Introduction, one possible explanation for our finding that $Ca^{2+}$-induced relaxation is intact in the $CB_1$ knockout mouse, yet is blocked by SR141716A, is that the blockade by SR141716A is mediated by nonspecific actions of the compound. As noted above, it has been reported that at high micromolar concentrations, SR141716A blocks $CB_2$ receptors,[8] gap junctions,[10] $Ca^{2+}$ entry channels,[10] and $K_{Ca}$ channels.[10] Our finding that SR144538, a selective $CB_2$ antagonist, had no effect on $Ca^{2+}$-induced relaxation in the $CB_1$ knockout animal indicates that SR141716A is not blocking the relaxation through a $CB_2$ receptor effect. Moreover, our finding that 18$\alpha$-glycyrrhetinic acid, a compound with gap junction–blocking activity,[10] has no effect on $Ca^{2+}$ relaxation in the rat makes it unlikely that SR141716A is attenuating $Ca^{2+}$-induced relaxation through a gap junction–blocking effect. It also seems unlikely that SR141716A is blocking $Ca^{2+}$-induced relaxation by inhibiting L-type $Ca^{2+}$ channels because L-type $Ca^{2+}$ channel inhibition is generally associated with a decrease in agonist-induced contraction and not blockade of a relaxation event.

In view of the fact that $Ca^{2+}$-induced relaxation is antagonized by the selective large conductance $K_{Ca}$ channel antagonist iberiotoxin,[4] we were particularly concerned about the contribution of possible $K^+$ channel–blocking activity of SR1416171A, as has been reported by White and Hiley.[9] To

directly address this issue, we determined the effect of a range of concentrations of SR141716A on whole-cell $K^+$ channel currents in isolated vascular smooth muscle cells. The results showed clearly that SR141716A causes concentration-dependent inhibition of these currents. Two findings are of particular interest for the present study. One is that 1 $\mu$mol/L SR141716A caused <8% inhibition of $K^+$ channel current, whereas the same concentration inhibited $Ca^{2+}$-induced relaxation by >60%. A second finding is that the effect of SR141716A is additive to the effect of TEA and iberiotoxin. This suggests that SR141716A inhibits $K^+$ channels other than $K_{Ca}$ channels, presumably voltage-dependent $K^+$ channels, which are less sensitive to SR141716A than the site at which SR141716A inhibits $Ca^{2+}$-induced relaxation. In view of these observations, it seems unlikely that the inhibitory effect of SR141716A on $Ca^{2+}$-induced relaxation is mediated by an inhibition of either $K_{Ca}$ or voltage-dependent $K^+$ channel activity.

A second possible explanation for our finding that $Ca^{2+}$-induced relaxation is intact in the $CB_1$ knockout mouse, yet is blocked by SR141716, is that SR141716A is blocking a nonclassical cannabinoid receptor. Kunos and colleagues[15] have recently demonstrated that abnormal cannabidiol, an analog of the nonpsychoactive marijuana-constituent cannabidiol,[23] causes relaxation of the isolated rat mesenteric bed. They further found that both anandamide and abnormal cannabidiol have mesenteric vasodilator activity in the $CB_1$ knockout mouse, and the effect of both compounds is antagonized by SR141716A or cannabidiol.[15] These findings have been interpreted to indicate that anandamide and other endocannabinoids can modulate vascular function by activating a nonclassical cannabinoid receptor. Of interest, in the present report, we show that O-1918, a novel analog of cannabidiol that blocks abnormal cannabidiol-induced relaxation in isolated rat and mouse mesenteric arteries (S. Batkai and G. Kunos, 2001, unpublished observations), blocks $Ca^{2+}$-induced but not acetylcholine relaxation in isolated mouse arteries. This observation provides further support for the hypothesis that $Ca^{2+}$-induced relaxation is mediated by sensory nerve dependent release of an endocannabinoid, possibly anandamide, which diffuses to underlying vascular smooth muscle and activates a specific anandamide receptor, which induces hyperpolarizing relaxation.

## Acknowledgments

We thank Dr Andreas Zimmer for providing the breeding pairs for the $CB_1$ receptor knockout mice and their controls and Dr Raj Razdan for the synthesis of compound O-1918. The authors acknowledge the expert secretarial assistance of Barbara Manning. This work was supported by grants from the National Institutes of Health: HL 54901, HL59868, and HL64761 (R.D.B.) and HL32469 (W.F.J.).

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